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14. ABSTRACT
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Fusing chlorophyll fluorescence and plant canopy reflectance to detect TNT contamination in soils

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ABSTRACT

TNT is released into the soil from many different sources, especially from military and mining activities, including buried land mines. Vegetation may absorb explosive residuals, causing stress and by understanding how plants respond to energetic compounds, we may be able to develop non-invasive techniques to detect soil contamination. The objectives of our study were to examine the physiological response of plants grown in TNT contaminated soils and to use remote sensing methods to detect uptake in plant leaves and canopies in both laboratory and field studies. Differences in physiology and light-adapted fluorescence were apparent in laboratory plants grown in N enriched soils and when compared with plants grown in TNT contaminated soils. Several reflectance indices were able to detect TNT contamination prior to visible signs of stress, including the fluorescence-derived indices, R_{740}/R_{850} and R_{735}/R_{850}, which may be attributed to transformation and conjugation of TNT metabolites with other compounds. Field studies at the Duck, NC Field Research Facility revealed differences in physiological stress measures, and leaf and canopy reflectance when plants growing over suspected buried UXOs were compared with reference plants. Multiple reflectance indices indicated stress at the suspected contaminated sites, including R_{740}/R_{850} and R_{735}/R_{850}. Under natural conditions of constant leaching of TNT into the soil, TNT uptake would be continuous in plants, potentially creating a distinct signature from remotely sensed vegetation. We may be able to use remote sensing of plant canopies to detect TNT soil contamination prior to visible signs.

Keywords: Chlorophyll fluorescence, hyperspectral reflectance, photosynthesis, plant physiology, soil N, trinitrotoluene, water relations

INTRODUCTION

Using vegetation as sentinels to indicate presence or absence of toxic contaminants is not new and could potentially provide a mechanism for large-scale detection, especially as advances in spectroscopic methods have improved our ability to remotely monitor and understand changes in vegetative canopies. Explosives have been released into the environment from munitions production and processing facilities, and as buried unexploded ordinance (UXOs). Explosives and associated metabolites in the soil are absorbed by roots and may induce a toxic effect on growth and biomass that varies among species[1,2]. Although plants are considered as indicators of the general environment where they grow, both morphological and physiological characteristics are a function of integrated responses to multiple environmental variables. Our goal is to use hyperspectral remotely sensed imagery coupled with chlorophyll fluorescence to detect soil contamination of explosives in field studies.

Numerous advances in fluorescence and reflectance spectroscopy have allowed for pre-visible stress detection in plants[3,4]. This research has been applied to many areas of environmental stress and is recently gaining attention as an innovative and unique method for detecting anthropogenic stress influences in the environment. With continued elucidation of plant responses to soil contaminants, it may be feasible to use naturally occurring vegetation to monitor the environment, particularly in regard to explosive compounds. This would provide a tremendous advantage for the military, including the detection of buried UXOs in abandoned war zones.

While numerous studies have examined uptake and biotransformation of various explosive compounds in plants[5,6,7], less attention has been given to the impact on photosynthetic processes and potentially changes in chlorophyll functioning as

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a result of anthropogenic stress. Xenobiotics that reach the leaves may be conjugated with other compounds and compartmentalized in the vacuole, cell wall or lignin[8], providing the potential ability to use various spectroscopic methods to detect stress to contamination. Little research has focused on hyperspectral reflectance changes in plants exposed to explosives, which could provide a new, inexpensive and highly significant method to detect explosives in the environment. Further, there is no apparent effort to distinguish among the effects of TNT and other forms of soil N. This is important because TNT undergoes nitroreduction and removal of N from the ring in plant cells. The specific objectives for our study were to experimentally evaluate the effects of various soil concentrations of TNT and corresponding N concentrations on plant physiological parameters relative and to link potential differences among treatments to measured changes in chlorophyll fluorescence and hyperspectral reflectance, and to link our laboratory experiments with field based measured at sites with suspected TNT contaminated soils and reference sites without contamination.

METHODS

Myrica cerifera L., Myricaceae, (wax myrtle) was chosen as the study species because the physiology and natural stress response has been well quantified and it occurs at field sites containing potential explosives contamination. Coastal military facilities are frequently inhabited with *M. cerifera*, and this could provide future field opportunities in contaminated soils. For laboratory studies, fruits of *M. cerifera* were collected from Hog Island (37° 40'N; 75° 40'W), a barrier island located on the Eastern Shore of Virginia, and crushed with a mortar and pestle to break the waxy coating and scarify seeds. Seeds were sown in transparent plastic trays filled with one inch of Perlite growth medium, and watered as necessary. Plants with at least three sets of secondary leaves were transplanted into 2 L plastic pots and grown for at least 5 months prior to experimentation. Plants were grown in a Conviron environmental chamber (CMP 3244, Controlled Environments Limited, Asheville, NC) under a photosynthetic photon flux density (PPFD) of approximately 700 μmol m^{-2} s^{-1}, 48% relative humidity, a photoperiod of 14 h, and a day/night temperature of 30/25 °C. Saplings were 50 cm at the beginning of the experiment and kept continually moist, but well-drained throughout the experiment.

Methods for TNT experiments are detailed in Naumann et al.[9]. For N treatments, 200 mL of water containing various concentrations of $Ca(NO_3)_2$ were mixed and homogenized with 4.2 kg of soil a low N soil to obtain initial concentrations of 47, 156, 390 and 780 mg of $Ca(NO_3)_2$ kg^{-1} dry soil. These concentrations represent the relative N concentration used in TNT experiments: 30, 100 250 and 500 mg TNT kg^{-1} dry soil, respectively.

Measurements of photosynthesis (A_{Net}), stomatal conductance to water vapor diffusion (g_s) and leaf pigment concentrations were compared across N and TNT treatments and through time. Rates of stomatal conductance and leaf net photosynthesis were measured using an infrared gas analyzer (LI- 6400, LI-COR Biosciences, Inc., Lincoln, NE). Leaf samples were collected by punching forty 0.32 cm^2 disks from each plant at the end of the experiment. Chlorophyll concentrations were determined based on methods recommended by Šesták[10]. Samples were then ground with a mortar and pestle, filtered, and analyzed using a Spectronic 21 spectrophotometer. Chlorophyll concentrations were calculated using equations described by Holm[11].

Table 1. Select vegetation indices used in our statistical analyses

Reflectance Index	Formula	Reference
Water Band Index (WBI_{970})	R_{970} / R_{900}	(12)
Chlorophyll Index (CI)	$(R_{750} - R_{705})/ (R_{750} + R_{705})$	(13)
Structural Insensitive Pigment Index (SIPI)	$(R_{800} - R_{445})/ (R_{800} + R_{680})$	(14)
Physiological Reflectance Index (PRI)	$(R_{531} - R_{570})/ (R_{531} + R_{570})$	(15)

Optical measurements of chlorophyll fluorescence and hyperspectral reflectance were also made on the fourth or fifth fully expanded leaf of each plant using a pulse amplitude modulated leaf chamber fluorometer (LI- 6400, LI-COR Biosciences, Inc., Lincoln, NE). The following ratios were calculated $\Delta F/ F'_m$ (quantum yield of photosystem II, PSII, in the light-adapted state) and F_v/F_m (maximum quantum use efficiency of PSII in the dark-adapted state). Measurements of leaf reflectance were taken concurrently with physiological and fluorescence measurements. An ASD FieldSpec Pro

reflectance radiometer (Analytical Spectral Devices, Inc., Boulder, CO) was used to measure the spectral reflectance of leaves between 350–2500 nm. To acquire a representative value, multiple spectra were collected and averaged for each leaf. A NIST spectralon reflectance standard was used as a white reference to optimize instrument gains prior to each canopy measurement. Using the resulting reflectance values, several reflectance indices examining potential changes in pigments, reflectance-derived fluorescence and water content were calculated (Table 1).

Variations in photosynthetic characteristics, stomatal conductance, fluorescence, reflectance indices and chlorophyll concentrations among treatment plants relative to control plants were analyzed analysis of variance[16] for each stress experiment. Dunnett's multiple comparisons[16] ($\alpha = 0.05$) identified significant differences in treatment plants relative to controls for laboratory studies.

Parallel field studies were conducted at the US Army Corps of Engineers Duck Pier Field Research Facility, Duck, North Carolina, USA. This 176-acre US Army Corps of Engineers facility is located on a barrier island along the Atlantic Coast and was once used as a bombing range by the US Navy. As such, there are numerous UXOs mapped across the site. The physiological measurements described for the laboratory portion of the study were also quantified in the field for *M. cerifera* shrubs growing in sites suspected of containing buried UXOs and reference sites considered clear of UXOs. Also similar to the laboratory experiment, optical measurements of chlorophyll fluorescence and hyperspectral reflectance were also obtained.

RESULTS AND DISCUSSION

TNT induced physiological stress in *M. cerifera* which was markedly different from similar N concentration additions as seen in stomatal conductance, photosynthesis and fluorescence measurements. For TNT experiments, decreases in stomatal conductance and photosynthesis occurred in all treatment plants by week 3 and remained low throughout the experiment[9] (Figure 1). Comparatively, similar concentrations of N induced a stress response by week 3 as seen in decreased photosynthesis in plants treated with 156, 390 and 780 mg of $Ca(NO_3)_2$ kg^{-1} dry soil ($F = 8.11$, $P = 0.000$), however by week 5 all plants recovered to control values except for the highest N concentration ($F = 9.42$, $P = 0.000$,).

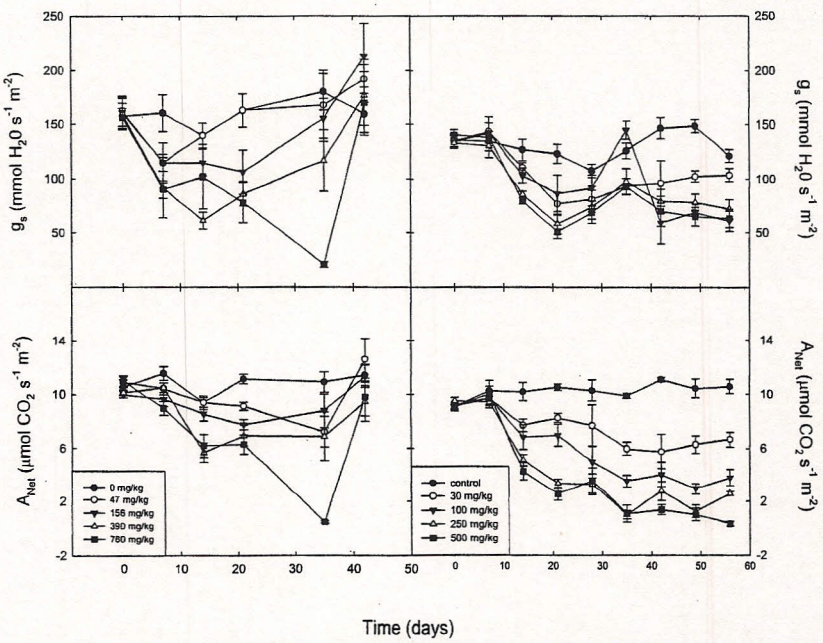

Figure 1. Variations in stomatal conductance (g_s) and net photosynthesis (A_{Net}) for *Myrica cerifera* shrubs growth over a range of soil N concentrations, $Ca(NO_3)_2$ on the left panel and TNT on the right. TNT data are from Naumann et al.[9]. Vertical bars denote ± 1 standard error of the mean.

Initial field measurements at Duck Pier showed lower values of stomatal conductance and photosynthesis at sites growing above potentially contaminated areas (Figure 2). Both sites had similar exposure and distance to the shoreline to eliminate variations in environmental stress; however these measurements are initial and more research is needed to confirm that changes are due to explosives contamination.

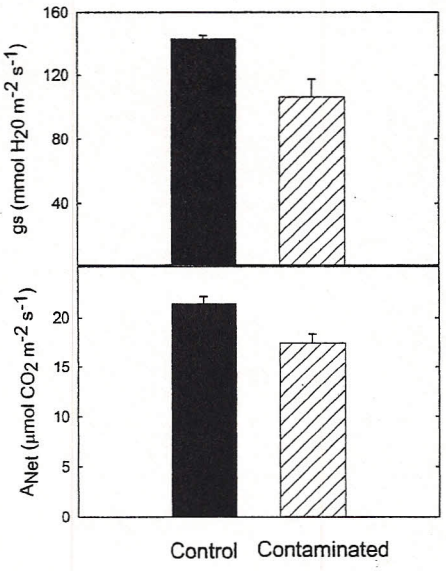

Figure 2. Stomatal conductance (g_s) and net photosynthesis (A_{Net}) for *Myrica cerifera* shrubs growing at references sites and on sites with potentially buried UXOs. Vertical bars denote one standard error of the mean.

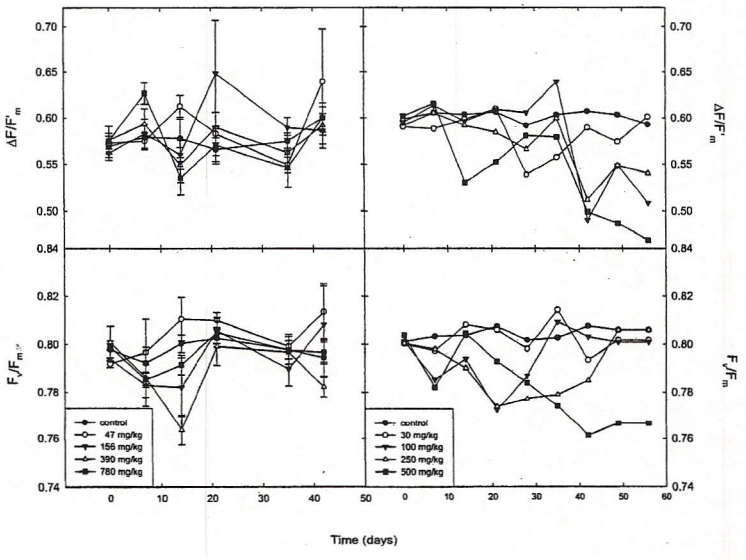

Figure 3. Variations in light adapted ($\Delta F/ F'_m$) and dark adapted (F_v/F_m) fluorescence through time for *Myrica cerifera* shrubs growth over a range of soil nitrogen concentrations, $Ca(NO_3)_2$ on the left panel and TNT on the right. TNT data are from Naumann et al.[9]. Vertical bars denote ± 1 standard error of the mean.

Responses in chlorophyll fluorescence for both N and TNT treatments were very different than those due to drought, flooding and salinity, where a steady decline was seen due to continuous exposure to stress[11, 12]. For plants treated with TNT, patterns were erratic, possibly due to differences in uptake from week to week. In plants treated with 100 mg kg^{-1} a positive effect on quantum yield of PSII was seen (Figure 3). Overall, reductions in chlorophyll fluorescence were seen in most TNT treated plants by week 6, and stayed lower than controls for the remainder of the experiment. For the higher TNT concentrations, reductions in $\Delta F/F'_m$ occurred by week 2, but changes in F_v/F_m were only seen in plants treated at 500 mg kg^{-1} by week 5[9]. Comparatively, changes in $\Delta F/F'_m$ and F_v/F_m were only seen during week 3 at 390 mg of $Ca(NO_3)_2$ kg^{-1} dry soil ($F = 2.99$, $P = 0.044$; $F = 5.20$, $P = 0.005$, respectively). Otherwise, there were no differences in fluorescence from additional N inputs. At Duck Pier, there were also no differences in chlorophyll fluorescence ($F = 0.76$, $P = 0.408$); however it is more difficult to assess in field situations as incident light and diurnal fluctuations are important, as well as exposure to other environmental stress. There were no significant differences in total chlorophyll at the end of TNT treatment[9], N treatment ($F = 0.43$, $P = 0.789$) or at between field sites at Duck Pier ($F = 0.09$, $P = 0.772$).

Table 2. Summary of ANOVA of leaf reflectance indices for week 6 of the TNT experiment, week 3 of the N experiment and the field study. *P* values are reported for significant differences among treatments. [a] refers to significant differences at 30, 100 and 500 mg TNT kg^{-1} soil. TNT data are from Naumann et al.[9].

Reflectance Index	TNT – 500 mg kg^{-1}	N – 780 mg kg^{-1}	Field
WBI	0.09	0.53	**0.00**
CI	0.06	**0.01**	**0.00**
SIPI	**0.04**	0.07	**0.00**
PRI	**0.02**	0.10	0.42
R_{685}/R_{655}	**0.03**	0.12	0.57
R_{680}/R_{630}	**0.04**	0.43	0.16
R_{735}/R_{850}	**0.03**	0.12	**0.00**
R_{740}/R_{850}	**0.04**	0.13	**0.00**
R_{761}/R_{757}	**0.00**[a]	0.06	**0.01**
D_{715}/D_{705}	**0.02**	**0.03**	**0.00**
D_{max}/D_{745}	**0.04**	0.07	**0.00**
D_{705}/D_{722}	**0.03**	**0.04**	**0.00**
R_{750}/R_{710}	**0.02**	**0.03**	0.16

Hyperspectral reflectance provided a unique insight to differences in laboratory treatments. Multiple reflectance indices and ratios were examined to look for potential indications of stress due to the various treatments. N additions had an apparent negative effect only during week 3 for plants exposed to 780 mg of $Ca(NO_3)_2$ kg^{-1} and those results are reported here (Table 2). By the end of the experiment, there were no differences in any reflectance indices, which was not the case in TNT experiments, where changes in reflectance indices remained. The reflectance results for the N experiments are supported by physiological measurements where treatment plants were only temporarily affected by higher N concentrations. *Myrica cerifera* is a N fixing species, and thus may be affected differently by additional N inputs relative to other species. There were numerous indices that indicated stress due to TNT exposure in laboratory studies, and some of these were also seen in field contaminated plants. Several reflectance-derived fluorescence indices indicated stress in laboratory treatments, while not all indicated stress in field sites. The most sensitive index to TNT induced stress was the ratio R_{761}/R_{757}, which detected stress at multiple concentrations[9], and showed differences in field contaminated sites (Table 2). Interestingly, the indices containing reflectance at 850 nm (in the near-infrared) indicated stress in TNT treated and field tested plants. Distinctions at 850 nm may be due to structural conformation changes in lignin and cell wall structure as TNT and metabolites become bound in the tissues. This index did not change under similar N concentrations. SIPI was lower in TNT treated and field contaminated plants which may be an indication of cell wall changes since it does include a near-infrared band. Aside from SIPI, pigment indices were variable among treatments. PRI, which is an indication of energy dissipation via the xanthophyll cycle, was only different for TNT treated plants. CI showed differences in N treated plants during week 3, but by the end of the experiment, there were no indications of varying chlorophyll content in reflectance or in extractions. CI was different in field sites, while

extractions did not reveal any differences. WBI indicated water stress at the field site and did not differ in either laboratory treatment. Some of the variability in lab studies compared to field studies can be explained by the type of exposure to contamination. In the field sites, exposure is chronic, from constant leaching of explosives into the soil, whereas laboratory studies are more acute, with a known concentration being taken up by the plants.

There is a need for technologies to remotely monitor movement of explosive compounds on active training ranges as well as to identify UXOs in the environment. Uptake of hazardous materials causes changes in chlorophyll content and biomass of plants, resulting from changes in the photosystem, as well as potential conformational changes within the cell wall. Remote sensing instrumentation is evolving into areas that make it possible to perform high resolution spectroscopic measurements in many regions of the electromagnetic spectrum. Until recently, these measurements were largely finite in spectral resolution and limited in the ability to measure direct chemical constituents that provide more information about a target. Initial studies indicate that hyperspectral reflectance remote sensing may be successful at establishing unique signatures related to exposure to toxic anthropogenic compounds; however more research is needed to fully evaluate the differences in optical signatures relative to natural environmental stress.

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